Correlation between Heart Disorders and Concentrations of Directly Measured Atrial Natriuretic Peptide in Plasma

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We used a new commercially available direct radioimmunoassay to measure human atrial natriuretic peptide (h-ANP) in plasma from 48 individuals who were being evaluated for left and right ventricular function. For 13 healthy individuals with normal ventricular function these concentrations ranged up to 54 ng/L. Measurements of h-ANP clearly differentiated between normal subjects, patients with coronary artery disease, and patients who had undergone orthotopic heart transplantation (ANOVA P < 0.0001, significant differences between all groups)—all showing normal ventricular function at rest. There was a strong negative correlation (r = -0.64, P < 0.001) between left ventricular ejection fraction and h-ANP concentrations in plasma of patients with proven coronary artery disease, patients with cardiomyopathy, and healthy individuals. Results by the present method and methods involving extraction of the sample correlated well. Evidently the direct assay of h-ANP in plasma yields information that could be used to help evaluate heart disorders and other pathophysiological conditions causing increased h-ANP concentrations in plasma.

Additional Keyphrases: radioimmunoassay · ventricular function · heart disease · organ transplants

Since 1981, the properties and physiological effects of human atrial natriuretic peptide (h-ANP) have been widely investigated, and its main natriuretic and vasodilative effects have been established (1). However, there is still controversy as to whether the concentrations of h-ANP in plasma indicate a primary pathophysiological role for this peptide in the development of hypertension (2, 3). Moreover, numerous different radioimmunoassays of plasma extracts have yielded various different values for the normal and pathophysiological ranges (1), indicating great methodological variability. To improve clinical practicability, investigators have tried to develop assays requiring no prior extraction (4). However, results for direct assays and those involving extraction correlated satisfactorily only in the higher pathophysiological range of concentrations, thus not allowing discrimination between healthy and diseased subjects (5). Some have doubted the reliability of direct assay (6) or have considered it too insensitive for clinical use (7, 8).

Therefore, we have used a commercially available direct assay kit for h-ANP to examine samples from different individuals, to assess its reliability for clinical use. To overcome the problems of increasing pathophysiological variations, we characterize the different patient groups in detail, in terms of their diagnosis and ventricular function.

Materials and Methods

Plasma Samples

A 4-mL specimen of blood was drawn into pre-chilled (with ice-cold water) evacuated tubes coated with dipotassium EDTA and, without delay, 0.1 mL (2000 kallikrein inhibition units) of aprotinin solution was added, and the samples were chilled in ice-cold water. Within 15 to 30 min the samples were centrifuged (30 min, 4 °C, 1700 × g). The plasma so obtained was divided into aliquots and frozen at −20 °C until assay.

Direct Radioimmunoassay of h-ANP

To measure the concentrations of ANP in plasma, we used a commercially available radioimmunoassay kit (Eiken Chemical Company Ltd., 33-8, Hongo 1-chome, Bunkyo-Ku, Tokyo, Japan) exactly as supplied. The kit contained rabbit antiserum to h-ANP,125I-labeled h-ANP; goat antiserum to rabbit IgG (second antibody); phosphate buffer (no detailed specification available); and alpha-h-ANP (1-28), 1280 ng/L, as standard. This standard was further diluted serially with the phosphate buffer to yield working standards having concentrations ranging from 20 to 640 ng/L.

The direct assay procedure (i.e., involving no sample extraction) was performed exactly as proposed by the manufacturer. The h-ANP concentration in the plasma samples was determined from the standard curve, which was prepared by using the values obtained from the serial dilutions of the standard (Figure 1).

For better characterization of the assay we also obtained a standard curve by serially diluting the supplied standard with h-ANP-free plasma prepared by extraction on Sep-Pak C-18 cartridges (9).

![Fig. 1. Standard curve for direct radioimmunoassay of ANP](image-url)

Fig. 1. Standard curve for direct radioimmunoassay of ANP

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<th>Concentration (ng/L)</th>
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Received July 13, 1988; accepted November 30, 1988.

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Two plasma samples were serially diluted and assayed, to assess the linearity of the relation between results and sample concentration.

We attempted to compare results by this direct assay with those by a well-known extraction assay described by Hartter et al. (9) for 30 plasma samples drawn from normal subjects, patients with coronary artery disease or cardiomyopathy, or patients who had undergone orthotopic heart transplantation. We also examined the correlation between the directly assessed h-ANP concentrations and the concentrations after prior extraction (9) by use of antibody supplied by Eiken Chemical Company. Samples were analyzed in duplicate by each method and the mean h-ANP concentration in plasma was only used if the 95% confidence ranges of each value overlapped; otherwise, the sample was excluded from further interpretation in this study. Therefore fewer than 30 identical samples were correlated for comparison of different methods, as indicated in the Results section.

The direct and extraction assays were done by two different and independent laboratories. Inter-laboratory correlation was assessed for the direct assay. The two laboratories were not informed as to sample donors’ health status or ventricular function.

Patients

The patients and controls (mean age 43, SD 15 y), all on a diet with salt unrestricted, were supine for 20 min before blood sampling. All patients were monitored for heart rate and blood pressure during the sampling. Simultaneously, precise scintigraphic assessment of left- and right-ventricular function allowed calculation of left- (LVEF) and right-ventricular ejection fraction, as described elsewhere (10). Significant valvular regurgitation was excluded in these subjects by evaluation of the regurgitant index (11).

Apparently healthy individuals, volunteers in an exercise-training program, were used as the normal controls to establish normal patterns for two-dimensional and Doppler echocardiography. Because the laws in our country forbid assessment of healthy individuals by scintigraphy, their normal echocardiographic heart function was assumed to represent a normal LVEF (mean 65%).

We investigated plasma h-ANP concentrations and ventricular function in 35 patients with the following diagnoses: definite coronary artery disease (17), cardiomyopathy (three), orthotopic heart transplantation (eight), and unexplained cardiac symptoms (seven).

Statistical Analysis

All data are expressed as mean ± SD; if appropriate, the range and median value are also provided.

The minimum h-ANP concentration clearly distinguishable from zero was established by using Student’s paired t-test, as applied by others (13). Ventricular function and concentrations of h-ANP in different patient groups were analyzed by both parametric and nonparametric tests (one-way analysis of variance: Student–Newman–Keuls test, and Kruskal–Wallis–ANOVA with Mann–Whitney U-test, respectively), so as not to be limited by the assumption of gaussian distribution. Linear regression analysis was also applied to differently defined patient subgroups.

Results

Radioimmunoassay

Figure 1 shows the characteristic shape of the standard curve for the radioimmunoassay without prior extraction. Half maximal displacement was 176 ± 13 ng/L. The sensitivity (detection limit) of the method was 20 ng/L at 95% and 40 ng/L at 99.9% confidence (paired t-test, n = nine assays of zero standards). Evaluation of the inter-assay variability for seven consecutive assays of h-ANP concentrations of 1.54 ± 8.2 and 41 ± 6.6 ng/L yielded CVs of 5.3% and 16%, respectively, indicating a sufficiently reproducible method. Inter-laboratory correlation was excellent (r = 0.986, SEE = 19 ng/L, n = 17).

No difference was seen between a dilution of pure standard prepared with h-ANP-free plasma and one prepared with buffer (Figure 1).

The inset in Figure 1 shows that two patients’ plasma samples with different supranormal h-ANP concentrations behaved similarly when further diluted by buffer.

Comparison of the direct assay with the assay after extraction yielded slightly contrasting results, depending on the antibody used. Figure 2a demonstrates that correlation with a previously validated method (9), performed by its developer, exhibited slight nonlinearity, as shown by polynomial fitting, whereas application of the same antibody as used in the direct assay to an otherwise identical extraction method developed by the same laboratory yielded excellent linear agreement, particularly in the critical low range of h-ANP concentrations in plasma (Figure 2b). These results indicate a problem with the reference method’s antibody sensitivity rather than with the specificity of the antibody used for the direct assay. This is discussed later also in the light of clinical aspects.

![Graph showing comparison between direct and indirect assay of h-ANP plasma concentrations](image-url)

Fig. 2. Comparison between direct and indirect assay of h-ANP plasma concentrations

(a) correlation between the Eiken (direct assay) and the comparison method (Nova after extraction) (9): r = 0.97, SEE = 38, y = 44 + 0.63x, n = 27.

(b) correlation between the Eiken method (direct assay Eiken) and the same antibody applied after prior extraction (Eiken after extraction): r = 0.99, SEE = 19, y = 3.6 + 1.06x, n = 27.

Inset to a: lower-range comparison of (a): —— linear regression line; - - - - polynomial fit. Arrow points to two patients who had had a myocardial infarction (one developed signs of congestion caused by supine posture).
Clinical Results

For samples from 13 healthy volunteers (mean age 30, SD 13 y) with defined normal ventricular function, plasma h-ANP concentrations ranged up to 54 ng/L (median 33, mean 29, SD 15). These values were compared with two differently diseased patient groups: eight patients after orthotopic heart transplantation (h-ANP: 184 ± 65 ng/L) and a subgroup of six patients with coronary artery disease also characterized by normal resting LVEF (h-ANP: 93 ± 29 ng/L). All three groups differed significantly from one another by both parametric and nonparametric tests ($F$-ratio = 38.6 and Chi-square = 20.1, respectively, $P < 0.0001$). Distinctive mean values and ranges are shown in Figure 3. It can be seen that h-ANP concentrations may vary significantly between patient groups with different diagnoses but with normal ventricular function. When h-ANP concentrations in plasma were compared with LVEF (right ventricular function was normal in all patient groups) by regression analysis, a significant linear negative correlation ($r = -0.64, P < 0.001$) between LVEF and plasma h-ANP concentrations was found after exclusion of patients in whom causes other than ventricular dysfunction could be contributing to increased h-ANP values (Figure 4b).

In the remaining, less-well-defined patients with non-specific cardiac symptoms or hypertension, and in patients after heart transplantation, there was no correlation at all ($r = 0.02$) between increased plasma h-ANP concentration and ventricular function (Figure 4a)—for reasons that are explained in the Discussion.

Discussion

Except for one other report (13), this study is the first in which the direct radioimmunoassay described above has been used systematically for clinical evaluation. Plasma samples were collected by clinicians who did not rely only upon clinical symptoms and diagnosis, but also assessed left and right ventricular function noninvasively and carefully supervised the plasma-collecting procedure. Because the plasma samples were subsequently assayed blind and in random order by two independent institutions whose main interest was the proper evaluation of the assay method itself, the results are likely to represent objective information about the assay’s methodological reliability as well as its potential usefulness in clinical practice.

Because no information about the exact composition of the kit was available from the manufacturer, we strictly followed the instruction for use, making no modifications. Evidently the kit will be particularly suitable for inter-institutional comparisons of clinical results, as indicated by the excellent correlation obtained for this method between the two cooperating radioimmunoassay laboratories.

However, a detection limit of 20 ng/L at 95% confidence was found, indicating less sensitivity than for the direct assay described by Marumo et al. (4). Nevertheless, the present method proved to be twice as sensitive as the direct method used by Jüppner et al. (7). The slightly higher coefficient of variation, 16% in the low normal range, was still acceptable, revealing h-ANP concentrations in plasma of normal controls similar to the normal values obtained with the highly sensitive direct assay described by Marumo et al. (4) and by others using specially developed indirect assays (5, 9, 12). We attribute this to the high specificity of the h-ANP antibody used, which leads to practically identical plasma h-ANP concentrations, unaffected by prior extraction, as shown in Figure 2b. These results were obtained by a laboratory that uses pre-analysis extraction of the sample and is known for its accuracy in h-ANP assessment, on the basis of an international collaborative study (14). However, when the same method was applied by the same laboratory using an accepted antibody as a reference to compare and establish compatibility with the direct assay, a slight nonlinearity was found, as demonstrated best by a polynomial fit in Figure 2a. Thus, the reference antibody applied tended to yield values in the upper range that were slightly too high and diminished values in the lower range of plasma h-ANP concentrations as compared with directly assessed values. This was in contrast to work by Richards et al. (12) and Yandle et al. (15), who, using a different h-ANP antibody, reported consistently higher h-ANP concentrations in plasma when such extraction was not performed. Therefore, the discrepancy between the direct and indirect (extraction) methods might be caused by different or changing sensitivity and (or) specificity of the antibody. However, when the antibody of the commercially available assay kit we tested was used, with or without prior extraction, no discrepancies could be found, as seen in Figure 2b. This suggests that the antibody is of adequate sensitivity and specificity, but this may apply only if aprotinin is added immediately after the sampling procedure to block the mechanism that leads to a nonspecific immunoreactivity, as

![Graph](image-url)
discussed elsewhere (9), and if the plasma is thoroughly centrifuged to eliminate the adverse effect of platelet contamination of plasma used in the direct assay, as has already been established (12). Nevertheless, this source of error was not eliminated in other important comparative studies of direct and extracted assays (12, 15). Our direct assay of plasma rendered platelet-free seemed insensitive to nonspecific immunoreactive components, which are thought to interfere with other direct assay methods, as described by Richards et al. (12). We attribute this to the apparently high specificity of the antibody used. Additional clinical data from the patient cohort investigated throw further light on the possibility of a higher sensitivity for this antibody. This is best demonstrated in two patients, indicated by the arrow in Figure 2a, for whom the direct assay yielded values in the pathological range, about three times higher than the values for plasma h-ANP concentration assessed in identical samples by the comparison method, which yielded values that clearly were within the normal range. Even after extraction, no such discrepancy existed with the antibody we tested. Even though some concentrations were outside the normal range, they appeared reasonable because the corresponding patients had documented myocardial infarction and slight congestive heart failure, respectively.

This new assay could just barely distinguish between plasma h-ANP concentrations of 20 ng/L and zero, so concentrations within this range could not be determined with sufficient confidence. Nevertheless, our normal values, which ranged up to 54 ng/L, agree well with those of Marumo et al. (4), obtained with a highly sensitive direct assay, and also with those evaluated after extraction (9, 12). However, they disagreed with the three-times-higher upper normal limit assessed by Jüppner et al. (7), who would appear to have used a less-specific and less-sensitive direct method.

Besides the specificity of the assay method, another explanation for the low upper limit of the h-ANP plasma concentrations in the control subjects might be that all controls were not merely apparently healthy, but had also been found normal in two-dimensional and Doppler echocardiography. However, one clinically healthy sportsman (30 years old) was excluded from the control group because of slight valvular regurgitation owing to a mitral valve prolapse. He also had an above-normal value for h-ANP, 90 ng/L (16). These precautions might not have been considered by authors using another direct method (3, 7), which has been criticized (9).

Comparison of defined groups with normal left and right ventricular function demonstrated for the first time that a freely available direct assay clearly discriminates between completely healthy individuals and patients with various underlying pathological conditions (discussed below). Although the healthy control group was significantly younger than the patients, the differences in h-ANP concentrations far exceeded the minimal difference attributable to age (12).

Patients having coronary artery disease with normal LVEF (normal systolic function) and patients with hypertension often suffer from ventricular diastolic filling abnormalities (17, 18) that result in slightly increased heart-chamber pressures and tensions, a cause of increased h-ANP secretion (19, 20). The assumption is further supported by recent reports about the localization of atrial natriuretic peptide immunoreactivity in the ventricular myocardium as well (21). Thus, in hypertensive patients with preserved systolic function (normal LVEF), hypertrophy of the left ventricle is ascribable to increasing myocardial mass, and this, mediated by the sympathetic stimulus of elevated myocardial wall stress, leads to increased production of h-ANP (22).

In patients who have had a myocardial infarction, increased ventricular h-ANP mRNA has been detected in infarcted as well as non-infarcted ventricles. Both hemodynamic and metabolic changes are thought to trigger the expression of the ANP gene (23), which precedes increased h-ANP production, and this might also explain the increased plasma h-ANP concentrations in patients with coronary artery disease when systolic function (LVEF) is still normal.

In patients who have undergone orthotopic heart transplantation, markedly increased h-ANP concentrations were found despite normal systolic ventricular function. Here, the changes in the electrolyte and fluid balance caused by corticosteroid intake (24) and the influence of other immunosuppressive drugs are thought to be the mechanism responsible for increased h-ANP secretion (25).

In our opinion, the groups of patients investigated here, with different underlying causes for h-ANP elevation, have adequately demonstrated the possible clinical relevance of the assay we tested in patients with normal systolic ventricular function, as assessed by left ventricular ejection fraction. However, with primary extracardiac causes of increased h-ANP production excluded, and for patients with defined presence or absence of cardiac disease, this study is the first to reveal a clear correlation between left ventricular dysfunction and elevated h-ANP as assessed by a direct assay (Figure 4b). Although no patient was symptomatic at the time of investigation, increased h-ANP production might also reflect increased pressures in the left ventricle and atrium (26, 27). However, in patients with less well-defined symptoms—comprising those with heart neuroses, mitral valve prolapse, hypertension, and unconfirmed coronary artery disease or orthostatic disorders of circulating volume regulation—ventricular function did not show any correlation (Figure 4a) with normal or above-normal plasma h-ANP concentrations, which might have been caused by cardiac or non-cardiac causes as discussed above.

This study shows that both cardiac and non-cardiac causes may influence h-ANP concentrations, which can be reliably measured by the present direct assay. Nevertheless, it must be stressed that, even if an accurate assay is used, evident clinical symptoms or diagnoses alone are an insuffcient basis for unequivocal interpretation of increased h-ANP concentrations. Thus, noninvasive testing of ventricular function usefully supplements the proper interpretation of h-ANP concentrations in apparently healthy individuals or patients.

We conclude that this direct assay of h-ANP in plasma yields clinically valuable information, which could be used to help evaluate heart disorders and other pathophysiological conditions causing increased plasma h-ANP concentrations. However, further extensive investigation of h-ANP concentrations in humans seems necessary for better understanding of its pathophysiological relevance. Such studies might be facilitated in practice by the availability of a sufficiently accurate direct assay method for h-ANP, as presented.

We thank Mrs. Renate Kargel, who skilfully carried out all the extraction assays, and Mrs. Ing. Elisabeth Legenstein, who was
involved in the analysis by the direct method. We also thank Miss
Gisela Krenauer, who performed the sampling procedure, and Miss
Elisabeth Pollirose and Mr. Farzad Eghbalian for their technical
assistance with heart scintigraphy. This work was kindly supported
by Pan Chemie Homburg Ges. m.b.H Wien.

References
1. Burnett Jr JC. Atrial natriuretic factor secretion: physiological
regulation and implications in cardiac vascular disease [Review].
levels of atrial natriuretic peptide in essential hypertension.
peptide and blood pressure in a geographically defined population.
radioimmunoassay of atrial natriuretic peptide (ANP) in human
5. Gutkowska J, Bonan R, Roy D, et al. Atrial natriuretic factor in
7. Jüppner H, Brabant G, Kapteina U, et al. Direct radioim-
unoassay for human atrial natriuretic peptide (hANP) and its
clinical evaluation. Biochem Biophys Res Commun 1986;139:1215–
23.
8. Singer DBJ, Sagnella GA, Markandu ND, et al. Atrial natriureti-
9. Hartzer E, Wołoszczuk W, Stummwoll HK. Radioimmunoassay
supine scintigraphic heart function and simultaneous conventional
exercise response in patients after myocardial infarction. Clin
of mild valvular regurgitation: its significance as assessed by
say for plasma alpha human atrial natriuretic peptide: a compar-
ison of direct and pre-extracted methods. J Hypertension
atrial natriuretic peptide in acute myocardial infarction [Letter].
Lancet 1987;i:1369.
14. Poole S, Das REG, Dzau VJ, et al. The international standard
for atrial natriuretic factor: calibration by international collabora-
tive study. Hypertension (in press).
15. Yandle TG, Espiner EA, Nicholls MG, et al. Radioimmuno-
assay and characterization of atrial natriuretic peptide in human
prolapse syndrome—stability of hyperadrenergic state, atrial natri-
uretic factor, and platelet abnormalities. In: Bourdoulas H, Wooley
CF eds. Mitral valve prolapse and the mitral valve prolapse
asynchronous left ventricular regional function and global ventricu-
diastolic filling in symptomatic compared to asymptomatic hyper-
plasma levels of immunoreactive atrial natriuretic hormone and
12.
atrial natriuretic peptide immunoreactivity in the ventricular
myocardium and conduction system of the human fetal and adult
natriuretic peptide and plasma catecholamines in arterial hyper-
23. Galipeau J, Nemec M, Drouin J. Ventricular activation of the
atrial natriuretic factor gene in acute myocardial infarction [Letter].
24. Saxenhofer H, Angst M, Weidmann P. Corticosteroid-induced
stimulation of atrial natriuretic peptide in man. Acta Endocrinol
(Copenhagen) 1968;118:79–86.
25. Singer DB, Buckley MG, MacGregor GA, et al. Raised concen-
trations of plasma atrial natriuretic peptides in cardiac transplant
elevation in congestive heart failure in the human. Science